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A novel technique for visualizing the intracellular localization and distribution of transported polyamines in cultured pulmonary artery smooth muscle cells

Shewan M. Aziz* [a], Mustafa Yatin [b], David R. Worthen [c], David W. Lipke [b], Peter A. Crooks [c,*]

[a] *Department of Pharmacy Services, Henry Ford Hospital, Detroit, MI 48202-2689, USA*
[b] *Division of Pharmacology and Experimental Therapeutics, College of Pharmacy, University of Kentucky, Lexington, KY 40536-0082, USA*
[c] *Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky, Rose Street, Lexington, KY 40536-0082, USA*

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Abstract

The use of a combination of monofluorescein adducts of spermidine (FL-SPD) and spermine (FL-SPM) with confocal laser scanning microscopy (CLSM) provides a useful means for monitoring the fate and time-dependent changes in the distribution of transported polyamines within living cells. Polyamine-fluorescein adducts were synthesized from fluorescein isothiocyanate and the appropriate polyamine. Monofluorescein polyamine adducts (ratio 1:1) were isolated using thin layer chromatography, and the structure and molecular weight of the monofluorescein polyamine adducts were confirmed using NMR and mass spectroscopy, respectively. The covalent linkage of the fluorescent adduct moiety to SPD and SPM did not influence their rate of uptake by bovine pulmonary artery smooth muscle cells (PASMC). Similar to ^{14}C-SPD and ^{14}C-SPM, the rate of uptake of ^{14}C-FL-SPD and ^{14}C-FL-SPM in PASMC was temperature-dependent. Treatment for 24 h with difluoromethylornithine (DFMO), a selective blocker of the enzyme ornithine decarboxylase and an inducer of the polyamine transport system, significantly increased the cellular uptake of ^{14}C-FL-SPD and ^{14}C-FL-SPM compared to that of control cells. When compared to control cells, treatment of PASMC with the pyrrolizidine alkaloid monocrotaline for 24 h also significantly increased the cellular uptake of ^{14}C-FL-SPD and ^{14}C-FL-SPM. On the other hand, 24 h treatment of PASMC with a polymer of SPM, a selective blocker of the polyamine transport system, or with free spermine, markedly reduced the cellular accumulation of ^{14}C-FL-SPD and ^{14}C-FL-SPM. After a 20-min treatment of PASMC with FL-SPD or FL-SPM, CLSM revealed that adduct fluorescence was localized in the cytoplasm of living cells. Treatment with DFMO increased the cytoplasmic accumulation of both FL-SPD and FL-SPM. In addition, the fluorescence observed in the cytoplasm of chinese hamster ovary cells (CHO) was significantly higher than that detected in the cytoplasm of their polyamine transport deficient variants (CHOMGBG). The results of this study provide the first evidence of the utility of a novel method for visualizing the uptake, distribution, and cellular localization of transported polyamines in viable cultured mammalian cells.

* Corresponding authors. Tel.: +1 606 2571718; fax: +1 606 2577585.

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1. Introduction

The polyamines putrescine (PUT), spermidine (SPD) and spermine (SPM) are a ubiquitous group of cationic molecules that play a central intracellular role in cellular growth, differentiation and neoplastic transformation [1–5]. Polyamines are necessary for increasing and regulating DNA, RNA and protein synthesis. Increases in intracellular polyamine content are driven principally by a coordinated interaction between de novo synthesis and transmembrane transport pathways [1–5]. De novo polyamine synthesis is governed in part by the activity of the first enzyme, ornithine decarboxylase (ODC), which catalyzes, in a rate-limiting step, the conversion of the amino acid ornithine to the diamine, PUT. PUT in turn is converted sequentially to SPD and SPM by the action of several other enzymes [1–4].

Along with de novo polyamine synthesis, cells from a variety of tissues, including tumor cells, transport polyamines across their membranes through temperature- and energy-dependent uptake processes [6–10]. Recently, we have shown that both cultured bovine pulmonary artery smooth muscle cells (PASMC) and rat aortic smooth muscle cells express two transporters; i.e. a 'non-selective' pathway which imports all three polyamines, and a 'selective' pathway that imports SPD and SPM only [9–11]. Similar studies have shown that several cancer cell lines also express a temperature-dependent polyamine transport system [12,13].

It is currently well recognized that along with the de novo polyamine biosynthetic pathway, the influx of extracellular polyamines into mammalian cells also plays an important role in the regulation of intracellular polyamine content [6,9,10]. This observation is confirmed by studies showing that the pharmacologic inhibition of cellular polyamine biosynthesis using difluoromethylornithine (DFMO), a specific enzyme-activated irreversible inhibitor of ODC, exerts a cytostatic rather than a cytotoxic effect [4,14]. DFMO's lack of a cytotoxic effect was attributed to the restoration of cellular polyamine content through the induction of a transmembrane polyamine transport system [15–17]. In vitro studies consistently show that DFMO causes large increases in transmembrane polyamine uptake [4,9]. Evidence provided from intact animal systems also suggests that polyamine transport is an important compensatory mechanism and, more importantly, suggests that interruption of polyamine uptake may be a potential target for antineoplastic drug therapy. Moreover, like the polyamine biosynthetic pathway, the cellular uptake of polyamines increases in response to treatment with stimuli promoting cell growth or differentiation [6].

The induction of the polyamine transport system has been shown to be essential for the development of several diseases, including chronic hypoxia-induced pulmonary hypertension and neoplastic transformation. The transport of polyamines is greater in tumor cells than in their normal counterparts [6,14]. We have recently synthesized a polymeric conjugate of spermine (poly-SPM) which selectively blocks the polyamine transport system in PASMC [11]. Our results have also demonstrated that poly-SPM blocks polyamine transport, depletes cellular polyamine content, and exerts cytotoxic effects in several cultured human cancer cell lines and their multiple drug resistant (MDR) variants [12,13]. Recently, we have shown that upregulation of the polyamine transport system, but not of ODC expression, is responsible for the increase in hypoxic lung polyamine content and for the subsequent development of pulmonary hypertension [9,18,19]. Our in vivo studies showed that chronic hypoxic pulmonary hypertension is associated with decreased lung ODC activity and increased uptake of PUT from the vascular compartment [18]. Similar to in vivo studies, PASMC cultured under hypoxic conditions show a reduced ODC activity and, more importantly, induction of both the non-selective and selective polyamine transporters [9]. In spite of these findings, which

demonstrate the importance of the transported polyamines in the development of these diseases, only sparse information is available regarding the uptake, disposition, binding sites and subcellular localization of transported polyamines.

In this study, we describe a novel technique which combines the use of monofluorescein adducts of spermidine (FL-SPD) and spermine (FL-SPM) in conjunction with confocal laser microscopy for visualizing the cellular distribution and localization of transported polyamines.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) with 1000 mg l^{-1} d-glucose, L-glutamine, 25 mM Hepes buffer, and 110 mg l^{-1} sodium pyruvate, trypsin-EDTA, and penicillin/streptomycin solution were purchased from Gibco (Grand Island, NY). Defined bovine calf serum (BCS) was supplied by HyClone (Logan, UT). Radiochemicals and related supplies were purchased from Amersham (Arlington Heights, IL). All plasticware used for tissue culture was obtained from Costar (Cambridge, MA). Phosphate-buffered saline (PBS), glutaraldehyde, fluorescein isothiocyanate (FITC) and other drugs and chemicals used in this study were purchased from Sigma (St. Louis, MO). Thin layer chromatography silica gel (20 × 20 cm, 250 μm, TLC) plates was purchased from Fisher Scientific (Atlanta, GA). Deuterium oxide (heavy water, D_2O) and tetramethylsilane (TMS) used in NMR spectroscopic studies were purchased from Aldrich Chemical Company (St. Louis, MO).

2.2. Pulmonary artery smooth muscle cell cultures

Bovine artery smooth muscle cells (PASMC) were cultured as described previously [9,11]. In brief, main pulmonary arteries were harvested from freshly-slaughtered cattle (generously provided by Dawson's Packing, Louisville, KY), immersed in cold (4°C) Pucks F6 solution supplemented with 300 U ml^{-1} penicillin and 0.3 mg ml^{-1} streptomycin, and transported to the laboratory. The arteries were opened longitudinally and the endothelium gently scraped away. Medial explants, approximately 2 mm^2, were dissected from the subintima and plated in culture flasks containing DMEM supplemented with 10% BCS, 100 U ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin. The PASMC were grown to confluence and propagated in culture. The culture medium was changed every 3–4 days. Cells were harvested using a 0.05% solution of trypsin. All experiments utilized cells from passages between 2 and 10. The smooth muscle phenotype of the cells was confirmed by the presence of smooth muscle-specific actin as evidenced by immunocytochemical analysis (data not shown). Cell counts were determined by hemocytometry. Cell viability was assessed routinely using trypan blue exclusion according to standard techniques.

2.3. Preparation of the SPM conjugate

The reduced polymeric glutaraldehyde conjugate of SPM was prepared as previously described [11–13]. Briefly, a solution (1 ml) of 1 mM SPM hydrochloride in 0.2 M phosphate-buffered saline (PBS) was added to 1 ml of 0.2 M PBS containing glutaraldehyde (3%) at pH 7. The mixture was incubated at 37°C for 1 h. $NaBH_4$ (10 mg) was added to reduce the imine functionalities and the reaction was left to stand at ambient temperature for 30 min. The reaction mixture was then dialyzed (lower molecular weight cutoff: 12.5–15 KDa) against 0.2 M PBS.

2.4. Preparation and isolation of the monofluoresceinated polyamine adducts

Fluoresceinated polyamines were prepared by adding a solution (3 ml) of 0.1 mM of either SPD HCl or SPM HCl solution in Na_2HCO_3/Na_2CO_3 buffer (pH 9–9.5) to 3 ml of 0.1 mM FITC solution in Na_2HCO_3/Na_2CO_3 buffer (pH 9–9.5). After vortexing for 5 min, the mixture was incubated overnight at 4°C. To isolate the monofluoresceinated polyamine adduct, a portion of the crude mixture (500 μl) was applied to a TLC plate, and separation was carried out using

ascending chromatography. The fluoresceinated polyamine spots were eluted with a solvent system consisting of 10 mM sodium phosphate buffer (pH-7):acetone (80:20 v/v) [20]. Fluorescence intensity of the eluted fractions was measured in an Aminco-Bowman spectrofluorometer at 324–520 nm. A standard curve representing a relationship between concentration and fluorescent intensity of FITC was generated using spectrophotometry. To increase the yield of the monofluoresceinated polyamines, the amount of the FITC and the specific polyamine were increased proportionally in the reaction mixture. ^{14}C-Polyamines were used in the synthesis of the monofluorescein ^{14}C-polyamine adducts. Several well-distinguished separated bands were detected on TLC for FL-SPD and FL-SPM, respectively. The percent yield of polyamine in each separated band of fluoresceinated polyamine was measured by dividing the measured radioactivity in each line by total radioactivity of ^{14}C-SPD or SPM added to the reaction mixture.

2.5. ^{1}H-NMR spectroscopy, spectrofluorometry and FAB-mass spectrometry

The ratio of the polyamine:FITC conjugation was determined by using either nuclear magnetic resonance spectroscopy (NMR) or by measuring the ratio of fluorescence intensity to radioactivity. NMR spectra of the monofluorescent polyamine adducts were recorded using a 400-MHz Varian-VH Model spectrometer (Varian Associates, Palo Alto, CA). ^{1}H-NMR spectra of conjugates were run in D_2O and were referenced to tetramethylsilane as external standard. The fluorescence intensity of each fluoresceinated polyamine sample was measured by spectrofluorometry. Radioactivity in the same sample was measured using liquid scintillation spectrometry. The molecular weights of FL-SPD and FL-SPM were determined using mass spectrometry. Mass spectrometric determinations were carried out on a Kratos MS50TA mass spectrometer equipped with a fast atom bombardment source. Spectra were acquired with a Kratos Model 0590 computerized data system. Glycerol was used as the sample supporting matrix.

2.6. Confocal laser scanning microscopy

The subcellular distribution of fluorescent polyamine conjugates in living PASMC was assessed using confocal microscopy (Molecular Dynamics, Sunnyvale, CA) [21]. The system includes a Sarastro 2000 Confocal Laser Scanning Microscope (CLSM), a Nikon Diaphot inverted microscope, argon laser, scanning unit, an electronics cabinet, a Silicon Graphics computer, color monitor and a Genesis optomagnetic mass storage system. Installed software allowed for 3-D reconstructions of optical sections (Volume Analysis Information System; VANIS). This state-of-the-art feature permits detection of fluorescent signals at specified distances through the vertical dimension of the cell. The Sarastro system is equipped with software that allows quantification of the intensity of two fluorochromes in defined areas of the specimens. Thus, it is possible to semiquantify levels of intracellular polyamines in subcellular compartments. FL-SPD or FL-SPM (1 μM) was added to PASMC and cells were incubated for 20 min, either at 37 or 4°C. The culture media was then aspirated, the cells were washed with serum free DMEM twice, and examined under CLSM at 480 nm. PASMC were seeded at a density of 75000 cells well^{-1} and cultured in DMEM supplemented with 10% BCS for 24 h. PASMC were rinsed with fresh, serum-free DMEM, and 1 ml of serum-containing DMEM was then added. The cells were then exposed to either DFMO (1 mM) or monocrotaline (MCT; 2.5 mM) for 24 h. The culture medium was aspirated and 1 ml of serum containing media was added. Subsequently, the appropriate ^{14}C-polyamine or ^{14}C-FL-polyamine was added to each well at a concentration of 1 μM. After a 20-min incubation at either 37 or 4°C, the media was aspirated, replaced with fresh media, and the fluorescence intensity inside the cells was assessed using CLSM. The operating conditions were as follows; objective, 60 ×; pinhole aperture, 50 μM; image size 1024 × 1024 pixels; pixel size 0.21 μm. Average pixel intensity was measured within each cell in the field and expressed in the relative units of DCF fluorescence.

2.7. Polyamine transport

Polyamine transport was assessed as previously reported [11,12]. Briefly, PASMC were seeded at a density of 75000 cells well^{-1} and cultured in DMEM supplemented with 10% BCS for 24 h. PASMC were rinsed with fresh, serum-free DMEM, after which 1 ml of serum-free DMEM was added to each well and the cells were allowed to acclimate for 1 h. Next, the appropriate ^{14}C-polyamine or ^{14}C-FL-polyamine was added to each well at a concentration of 1 μM, and the cells were incubated for 20 min. At the appropriate time, media containing residual ^{14}C-polyamines was aspirated, cells were placed on ice, and rinsed with ice cold PBS. The PASMC were then digested for 1 h at room temperature in 1 N NaOH. Before determination of cell-associated radioactivity, 400 μl of the cell digest was neutralized with 400 μl 1 N acetic acid. An additional 400 μl distilled H_2O and 2 ml scintillation cocktail were added to the neutralized digests, and radioactivity was determined using a Packard liquid scintillation counter. The specific component of polyamine uptake was calculated by subtracting the polyamine uptake rate values at 4°C from those generated at 37°C at each polyamine concentration. Protein content was determined using an adaptation of the method described by Bradford [22]. The ^{14}C-polyamine content and uptake rate was normalized in terms of cellular protein content. To determine the effect of DFMO (1 mM) or MCT (2.5 mM) on the uptake of FL-polyamine by PASMC, cells were incubated with one of these agents for 24 h. Media was then aspirated and the polyamine transport assay with FL-polyamine adducts was conducted. For the competition studies, the specific competitor (free SPM or poly-SPM) was added to the culture media 1 min prior to the conduction of the polyamine transport assay.

2.8. Statistical analysis

Data are presented as the mean ± S.E.M. The significance of the differences between mean values was assessed using a one-way analysis of variance combined with Neumann-Kuels test or using an unpaired t-test, depending on the experimental design. P values ≤ 0.05 were considered to denote statistical significance.

3. Results

3.1. Results of ^{1}H-NMR spectroscopy, spectrofluorometry and FAB-mass spectrometry

Results of the Mass Spectrometry confirmed the molecular weights of the monofluoresceine polyamine adducts. The ratio of the polyamine:FITC conjugation was determined using ^{1}H-NMR Spectroscopy. The D_2O ^{1}H-NMR spectrum of FL-SPD showed resonances at δ 7.82–5.86 (m, 10 H, aromatic protons); 3.00–2.10 (m, 6 H, $3 \times CH_2-N+$); 1.92 (s, 1H, CH) and 1.80–0.80 (m, 8 H, $3 \times C-CH_2-C$, $1 \times CH_2-N-C=S$) ppm. The D_2O ^{1}H-spectrum of FL-SPM showed resonances at δ 7.65–5.85 (m, 10 H, aromatic protons); 2.95–2.00 (m, 10 H, $5 \times CH_2-N+$); 1.92 (s, 1 H, CH); and 1.78–0.80 (m, 10 H, $4 \times C-CH_2-C$, $1 \times CH_2-N-C=S$) ppm.

3.2. Separation and isolation of monofluoresceinated polyamines

The chromophoric properties of fluoresceinated polyamines render their detection on the TLC plate an easy operation. Three and four distinct, well-separated bands were detected on the TLC for FL-SPD and FL-SPM, respectively. The colored bands on TLC were scraped off with a razor and each band was collected in a 15-ml centrifuge tube. The silica gel matrix containing each band was vortexed three times with 5 ml of deionized water for 1 h each. The mixture was centrifuged and the supernatant collected and concentrated under evaporation with a stream of nitrogen gas. In both FL-SPD and FL-SPM mixtures, the top band (the fastest moving band, band 1) represented unreacted fluorescein isothiocyanate, as determined by measuring ^{14}C-SPD or ^{14}C-SPM radioactivity and fluorescence intensity in each TLC band using liquid scintillation spectroscopy and spectrofluorometry, respectively. The *Rf* values for the top band of both FL-SPD and FL-

SPM were 0.9. The presence of polyamine in each TLC band was also detected using ninhydrin staining. The other two lower moving bands in the FL-SPD mixture were presumed to be mono-substituted and disubstituted FL-SPD. The ratio of FL to SPD was 1 ± 0.03 and 1.9 ± 0.03 in bands 2 and 3, respectively. The slowest moving band ($Rf = 0.69$, band 3) was a single colored band almost at the origin of the TLC plate, which did not react with ninhydrin. Band 2 was also a single colored band ($Rf = 0.8$) and reacted with ninhydrin, indicating the presence of a primary amino group. In the FL-SPM mixture, the second band ($Rf = 0.79$) was presumed to be the mono-substituted SPM as the ratio of FL to SPM was 1 ± 0.05. The single colored band 2 also reacted with ninhydrin indicating the presence of a primary amino group. The other two bands for FL-SPM were presumed to be disubstituted and trisubstituted FL-SPM. The ratios of FL to SPM were 1.8 ± 0.03 and 2.7 ± 0.04 in peaks 3 and 4, respectively. Bands 3 and 4 also did not react with ninhydrin and both showed single ultraviolet spots at *Rf* values of 0.69 and 0.2, respectively. To determine the stability of the isolated monofluorescent polyamine adducts, a sample of either monofluorescent SPD or SPM (ratio 1:1) was run on TLC every 3 days for 2 weeks after isolation. Our results demonstrated that the monofluorescent polyamine adducts were stable and showed a single colored band on silica gel TLC plates for at least 2 weeks after their synthesis.

3.3. ^{14}C-FL-SPD and SPM transport as compared to ^{14}C-polyamine

As shown in Table 1, the addition of a fluorescent moiety did not affect the transport of the resulting conjugated polyamine in PASMC. The rate of uptake of ^{14}C-FL-SPD or ^{14}C-FL-SPM was not significantly different from that of non-fluorescent ^{14}C-SPD or ^{14}C-SPM, respectively. Similar to ^{14}C-SPD and ^{14}C-SPM [9], the uptake of FL-SPD and FL-SPM were temperature-dependent. At 4°C the uptake of FL-SPD or FL-SPM was significantly lower than that observed at 37°C (Table 2, Figs. 1 and 2). Trypan blue exclusion studies showed that the incubation of these FL-polyamines with PASMC for 1 h at concentrations from 0.1 to 10 μM did not influence cell viability.

Table 1
The rate of uptake of ^{14}C-polyamine and ^{14}C-monofluorescein-polyamine adducts (^{14}C-FL-polyamine) in cultured bovine pulmonary smooth muscle cells (PASMC)

Polyamine[b]	Polyamine uptake rate pmol min^{-1} mg^{-1} protein[a]
^{14}C-spermidine	7.5 ± 0.22
^{14}C-FL-spermidine	7.2 ± 0.30
^{14}C-spermine	4.57 ± 0.60
^{14}C-FL-spermine	4.4 ± 0.20

[a] Mean ± S.E.M. of eight experiments.
[b] PASMC were incubated with either ^{14}C-polyamine (1 μM) or ^{14}C-FL-polyamine adducts (1 μM) for 20 min at 37°C.

3.4. Effect of DFMO and MCT on ^{14}C-FL-SPD and SPM uptake in PASMC

We have previously shown that the treatment of cultured smooth muscle cells with DFMO (1 mM) for 24 h significantly increased the rate of uptake of ^{14}C-polyamines as compared to those determined in control cells [4–10,13,26]. Similarly, treatment of PASMC with 1 mM DFMO for 24 h upregulated the rate of ^{14}C-FL-SPD and ^{14}C-FL-SPM uptake by several-fold compared to that

Table 2
The effect of temperature on the rate of uptake of ^{14}C-monofluorescein-polyamine adducts (^{14}C-FL-polyamine) in cultured bovine pulmonary smooth muscle cells (PASMC)

Temperature (°C)[b]	Polyamine uptake rate pmol min^{-1} mg^{-1} protein[a]	
	^{14}C-FL-spermidine	^{14}C-FL-spermine
37	7.9 ± 0.4[c]	5 ± 0.6*
4	0.9 ± 0.2	1 ± 0.2

[a] Mean ± S.E.M. of five experiments.
[b] PASMC were incubated with either ^{14}C-polyamine (1 μM) or ^{14}C-FL-polyamine adducts (1 μM) for 20 min at 37 and 4°C.
*$P < 0.05$ 37°C vs. 4°C.

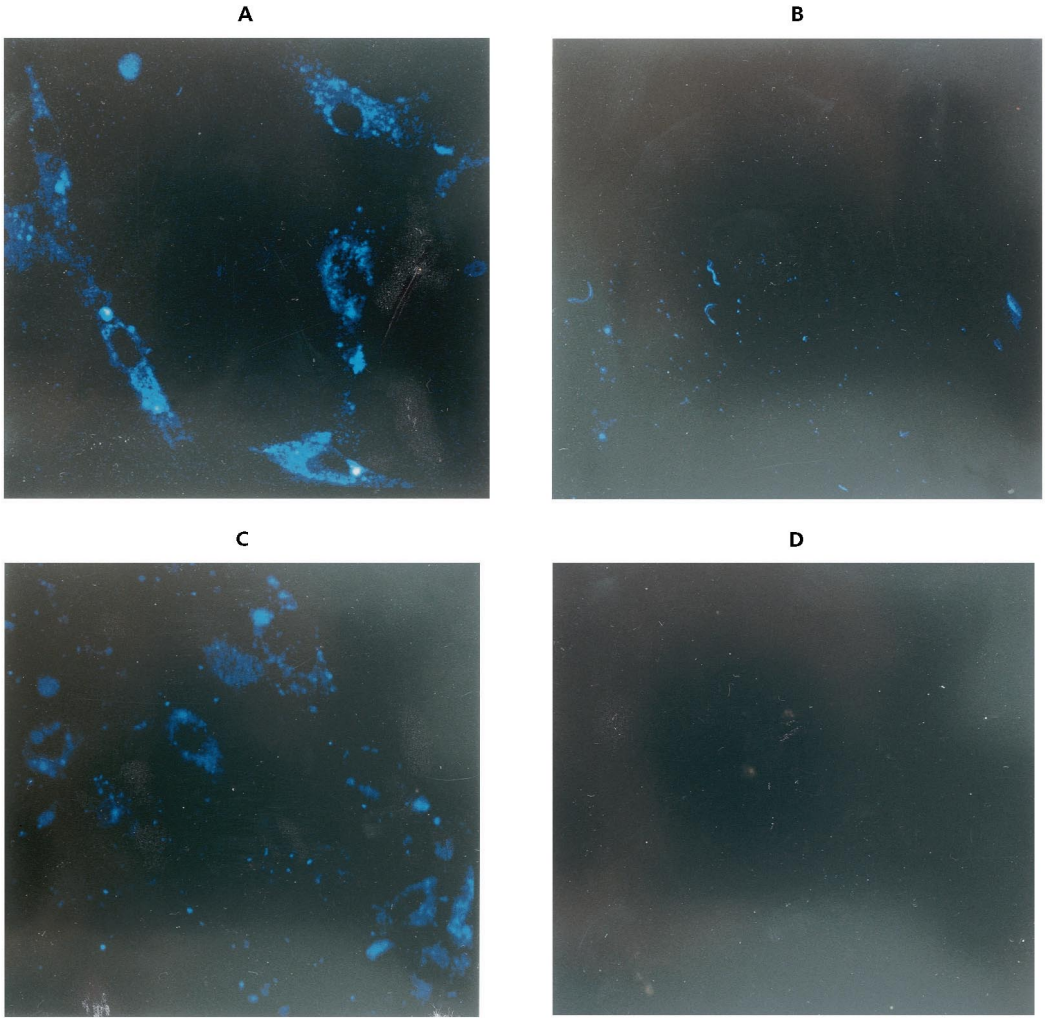

Fig. 1. Photomicographs from confocal scanning microscopy visualizing fluorescence intensity as DCF fluorescence emission. (A) PASMC incubated with FL-spermidine (1 μM) at 37°C for 20 min; (B) PASMC incubated with FL-spermidine (1 μM) at 4°C for 20 min; (C) PASMC incubated with FL-spermine (1 μM) at 37°C for 20 min; (D) PASMC incubated with FL-spermine (1 μM) at 4°C for 20 min. Blue color on the pseudocolor scale reflects the fluorescent intensity.

determined in control cells (Table 3 and Fig. 3). As shown in Table 3, the exposure of cultured pulmonary vascular cells to MCT (2.5 mM) for 24 h also increased the rate of uptake of ^{14}C-FL-SPD and ^{14}C-FL-SPD when compared to that determined in controls untreated. These results are in agreement with those previously generated with cultured pulmonary vascular cells [26].

3.5. Competition studies

The effect of the presence of increasing concentrations of free SPM in the media on cellular uptake of ^{14}C-FL-SPD and ^{14}C-SPM was examined. As shown in Table 4, unlabeled SPM in a dose-dependent manner blocked the rate of uptake of both ^{14}C-FL-SPD and ^{14}C-FL-SPM. In

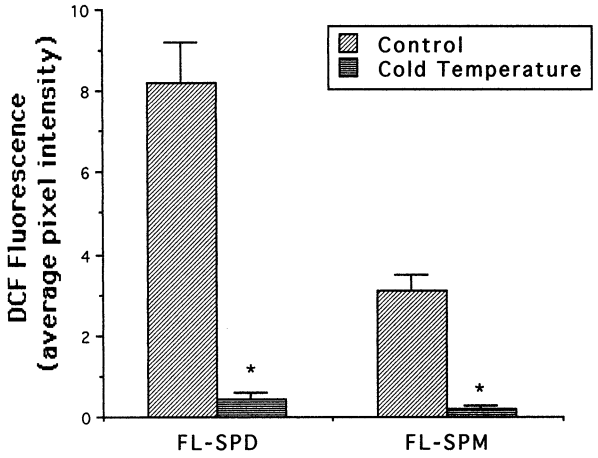

Fig. 2. Effect of temperature on DCF fluorescence of FL-polyamines reflected as average pixel intensity in bovine pulmonary artery smooth muscle cells (PASMC). Each column represents mean ± standard error of nine observations. *Significantly ($P < 0.05$) different from PASMC incubated at 37°C.

addition, the effect of pretreatment with FL-SPD or FL-SPM on the uptake of ^{14}C-SPD or ^{14}C-SPM was also determined (Table 5). As expected, FL-SPD (50 μM) reduced the uptake of ^{14}C-SPD by 15-fold. FL-SPM was also effective and decreased the uptake of ^{14}C-SPM by approximately 3-fold.

Table 3
Effect of pretreatment with DFMO or monocrotaline (MCT) on the rate of uptake of ^{14}C-monofluorescein-polyamine adducts (^{14}C-FL-polyamine) in cultured bovine pulmonary smooth muscle cells (PASMC)

Group[b]	Polyamine uptake rate pmol min^{-1} mg^{-1} protein[a]		
	Control	DFMO (1 μM)	MCT (2.5 mM)
^{14}C-FL-spermidine	7 ± 0.3	84.7 ± 7.6*	28 ± 0.73*
^{14}C-FL-spermine	4.9 ± 1	38.2 ± 1.6*	9.81 ± 1*

[a] Mean ± S.E.M. of six experiments. Cells were treated with either DFMO (1 mM) or MCT (2.5 mM).
[b] PASMC were incubated with ^{14}C-FL-polyamine adducts (1 μM) for 20 min.
*$P < 0.05$ control vs. treatment.

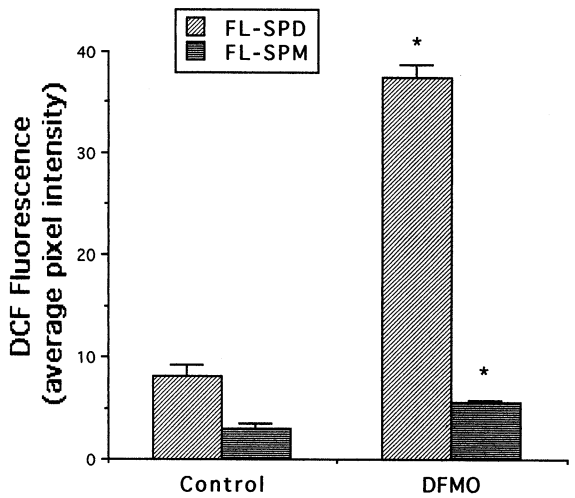

Fig. 3. Effect of difluoromethylornithine (DFMO) on DCF fluorescence of FL-polyamines reflected as average pixel intensity in bovine pulmonary artery smooth muscle cells (PASMC). Each column represents mean ± standard error of nine observations. *Significantly ($P < 0.05$) different from control PASMC.

3.6. Influence of poly-SPM on ^{14}C-FL-SPD and SPM uptake

The effect of treating cells with the selective inhibitor of the polyamine transport system, poly-SPM, on ^{14}C-FL-SPD or ^{14}C-FL-SPM uptake was also examined. Our previous studies showed that the addition of poly-SPM (20 μM) to PASMC selectively blocked the transport of the three polyamines, PUT, SPD and SPM [11]. Similarly, treatment with poly-SPM (20 μM) blocked the rate of uptake of both ^{14}C-FL-SPD and ^{14}C-FL-SPM by 18- and 11-fold, respectively (Table 4).

3.7. Confocal laser scanning microscopy to visualize intracellular localization of transported polyamines

The subcellular distribution of fluorescent polyamine conjugates in living PASMC was assessed using confocal microscopy. Fig. 1 shows representative photomicrographs visualizing fluorescence in cells exposed to either FL-SPD or FL-SPM (1 μM). A blue color on the pseudocolor

Table 4
The impact of poly-SPM and increasing concentrations of unlabeled spermine (SPM) on ^{14}C-monofluorescein-polyamine adducts (^{14}C-FL-polyamines) uptake rate in cultured bovine pulmonary artery smooth muscle cells

Treatment[c]	Polyamine uptake rate pmol min^{-1} mg^{-1} protein[a]	
	^{14}C-FL-spermidine[b]	^{14}C-FL-spermine[b]
Control	7.6 ± 0.3	4.7 ± 0.1
+SPM		
20 μM	4.1 ± 0.2*	1.36 ± 0.04*
50 μM	1 ± 0.001*	0.77 ± 0.074*
100 μM	0.17 ± 0.003*	0.37 ± 0.074*
+Poly-SPM		
20 μM	0.42 ± 0.0004*	0.4 ± 0.001*

[a] Mean ± S.E.M. of six experiments.
[b] PASMC were incubated with ^{14}C-FL-polyamine adducts (1 μM) for 20 min at 37°C.
[c] Poly-SPM or free SPM was added to PASMC 1 min before the conduction of the polyamine transport assay.
*$P < 0.05$ control vs. treatment.

scale reflects the fluorescence intensity. These photographs provide visual proof for the cellular localization of the fluorescence intensity after 20 min treatment with either FL-SPD or FL-SPM. It is clear from these photographs that, 20 min after treatment with either FL-SPD or FL-SPM, the highest fluorescence intensity is localized in the cytoplasm of the cells (Fig. 1). In addition, the cellular accumulation of either FL-SPD or FL-SPM is higher in PASMC incubated at 37°C than in cells incubated at 4°C (Figs. 1 and 2). Using confocal microscopy we were able to quantify the fluorescence intensity in the subcellular compartments. Fig. 3 shows DCF fluorescence as average pixel intensity. Relative to control cells, treatment with DFMO (1 mM) for 24 h significantly increased the fluorescence intensity of both FL-SPD and FL-SPM in the cytoplasm of the cells.

Table 5
The impact of monofluorescein-spermidine adducts (FL-spermidine) on the ^{14}C-polyamine uptake rate in cultured bovine pulmonary artery smooth muscle cells

Treatment[b]	Polyamine uptake rate pmol min^{-1} mg^{-1} protein[a]	
	^{14}C-spermidine	^{14}C-spermine
Control	7.6 ± 0.3	4.8 ± 0.2
FL-spermine (50 μM)[c]	—	1.6 ± 0.01
FL-spermidine (50 μM)[c]	0.51 ± 0.9	—

[a] Mean ± S.E.M. of six experiments.
[b] PASMC were incubated with ^{14}C-polyamine (1 μM) for 20 min.
[c] FL-spermidine or FL-spermine was added to PASMC 1 min before the conduction of the polyamine transport assay.

3.8. Cellular accumulation of FL-polyamines in chinese hamster ovary (CHO) and CHOMGBG cell lines

To further examine the selectivity of FL-polyamines for the polyamine transport system, CSLM was used to determine the subcellular distribution of these conjugated polyamines in living CHO cells and in their polyamine transport system deficient mutants, CHOMGBG cells. Fig. 4 shows representative photomicrographs visualizing fluorescence intensity in CHO and CHOMGBG cells exposed to either FL-SPD or FL-SPM (1 μM). Similar to the study conducted in PASMC, there is a high fluorescent intensity localized in the cytoplasm of CHO cells, but not in CHOMGBG cells. Fig. 5 shows DCF fluorescence as average pixel intensity for all treatment groups. The fluorescence intensity in the cytoplasm of the CHO cells was significantly higher than that detected in the cytoplasm of the polyamine transport system deficient mutant CHOMGBG cells.

4. Discussion

The results of this study provide the first insight into the successful use of monofluorescent polyamines in conjugation with confocal scanning microscopy (CLSM) to visualize the cellular localization, distribution and movement of these natural substrates inside living mammalian cells. The results of this study demonstrated that the monofluorescent polyamines, FL-SPD and FL-

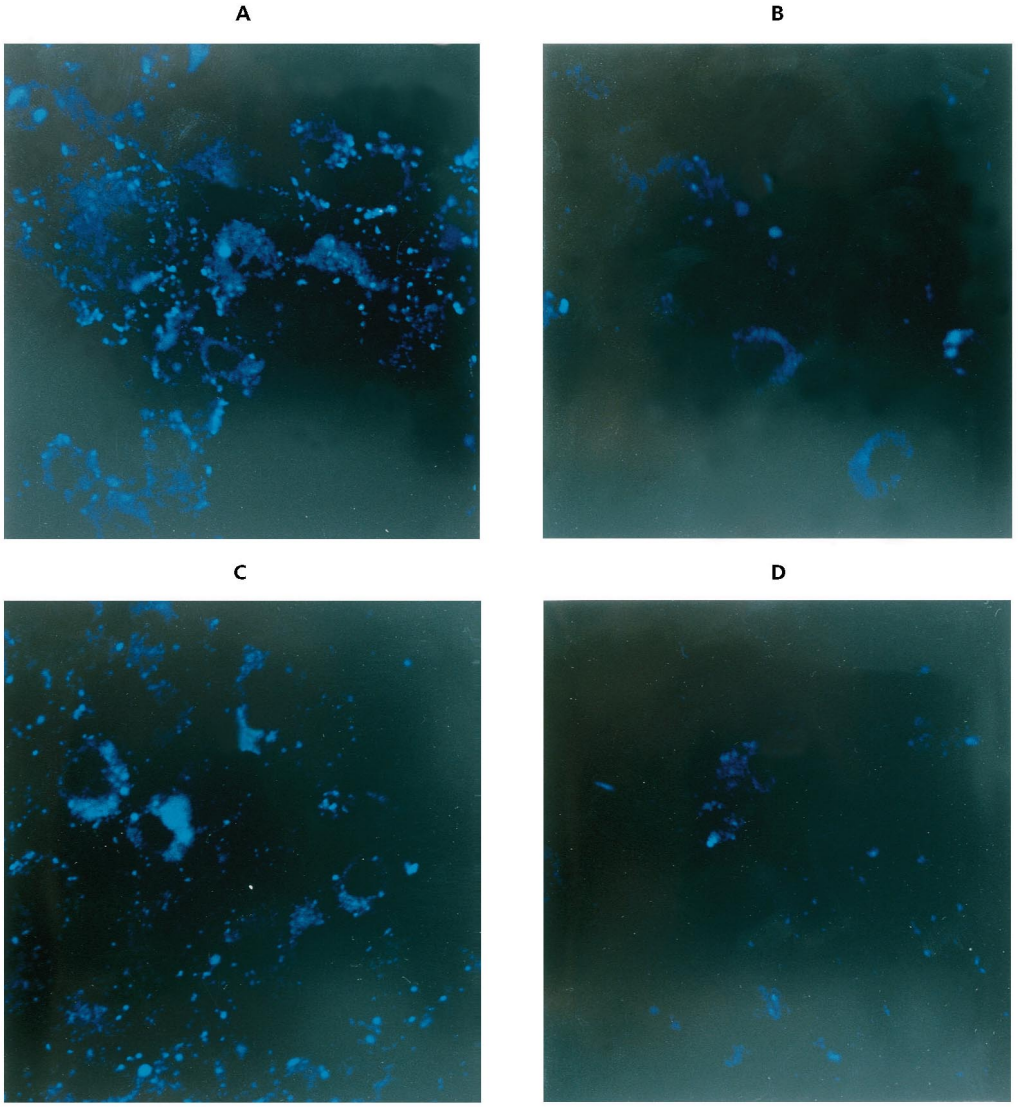

Fig. 4. Photomicographs from confocal scanning microscopy visualizing fluorescence intensity as DCF fluorescence emission. (A): chinese hamster cells (CHO) incubated with FL-spermidine (1 μM) at 37°C for 20 min; (B): polyamine transport system deficient cells (CHOMGBG) incubated with FL-spermidine (1 μM) at 37°C for 20 min; (C): CHO incubated with FL-spermine (1 μM) at 37°C for 20 min; (D): polyamine transport system deficient cells (CHOMGBG) incubated with FL-spermine (1 μM) at 37°C for 20 min. Blue color on the pseudocolor scale reflects the fluorescent intensity.

SPM, are biologically equivalent to their respective non-labeled polyamines in terms of their non-toxicity to smooth muscle cells and their competition for specific transmembrane polyamine transport systems. CLSM revealed that after a 20-min treatment with either FL-SPD or FL-SPM, the fluorescent intensity was localized in the cytoplasm of PASMC.

Our initial studies demonstrated that FL-SPD and FL-SPM conjugates were stable for at least 2 weeks after their synthesis. By using NMR spectroscopy, and determining the ratio of FITC

fluorescence intensity to ^{14}C-polyamine radioactivity, we were able to identify and isolate the monofluorescent polyamines (ratio 1:1). Positive ninhydrin staining indicated the presence of a primary amine in these monofluorescent polyamine adducts. Mass spectroscopic analysis further confirmed the identity of isolated monofluorescent polyamines. As determined by the trypan blue exclusion technique, the viability of PASMC treated for 1 h with these FL-polyamines at concentrations ranging from 0.1 to 10 μM did not differ significantly from that of untreated controls.

We next examined whether or not the FL-SPD or FL-SPM used in CLSM analyses were biologically equivalent to their respective non-labeled polyamines in terms of competition for specific transmembrane polyamine transport systems. Given the important function of the amino moieties in the polyamine structure with respect to their biological activity, linking a fluorescent molecule of modest size to a primary amine moiety on the polyamine might markedly perturb their normal biological activity. The results of our studies clearly demonstrate that monofluoresceinated polyamine adducts are equivalent to unlabeled polyamines in terms of their transport by the polyamine transport system. The competition study showed that unlabeled polyamine blocked the uptake of FL-SPD and FL-SPM in a dose-dependent manner. Analogously, the FL-polyamines were also effective in blocking the uptake of non-labeled ^{14}C-polyamine. These results indicate specificity of the FL-polyamines for the polyamine transport system.

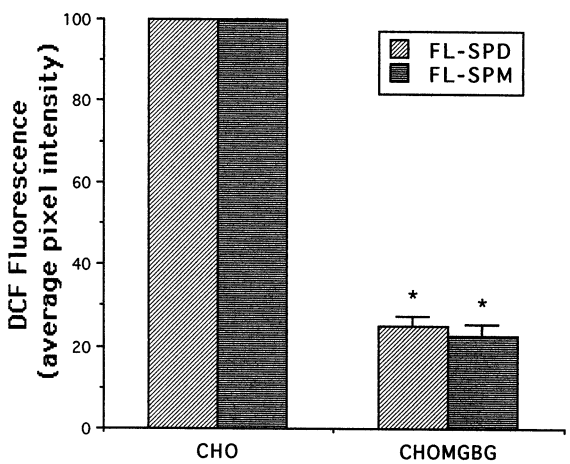

Fig. 5. DCF fluorescence of FL-polyamines reflected as average pixel intensity in chinese hamster cells (CHO), and their polyamine transport system deficient cells, CHOMGBG. Each column represents mean ± standard error of nine observations. *Significantly ($P < 0.05$) different from CHO cells.

We have used several other approaches to further examine the specificity of the FL-polyamines for the polyamine transport system. It has been demonstrated that in many mammalian cultured cells, the inhibition of endogenous polyamine synthesis using the selective blocker of ODC, difluoromethyl ornithine (DFMO), leads to a several-fold upregulation in the activity of the polyamine transport system [4,9]. We have found that a 24-h pretreatment of cells with DFMO caused significant increases in both PUT and SPD transport rates in several cancer cell lines and in other mammalian cells, including aortic smooth muscle cells [9–13]. In this study we have shown that treatment of cells with DFMO for 24 h also increased the uptake of FL-SPD and FL-SPM by several-fold.

It has been shown that the remodeling of the pulmonary vasculature induced by the alkaloid monocrotaline (MCT) is dependent on increases in lung polyamine content, which is partially mediated by upregulation of ODC activity [23,24]. Treatment with DFMO and the depletion of cellular polyamines significantly abolished MCT-induced pulmonary hypertension. In cultured pulmonary vascular cells, MCT markedly increased ^{14}C-SPD import in a dose-dependent fashion [25]. In this study, we examined the effect of treating PASMC with MCT (2.5 mM) on the rate of uptake of ^{14}C-FL-SPD and ^{14}C-FL-SPM. As was demonstrated in our studies with unlabeled polyamines, treatment of cells with MCT for 24 h also significantly increased the uptake of both ^{14}C-FL-SPD and ^{14}C-FL-SPM.

Recently, we have utilized a novel polyamine homopolymer, poly-SPM [11–13], to test the hypothesis that polyamine transport is a rational pharmacologic target in cancer therapy. Our results have demonstrated that poly-SPM is a specific blocker of the polyamine transport system in

mammalian cells. Further, co-treatment with DFMO markedly enhanced both the depletion of intracellular polyamine content and the cytotoxic effect induced by poly-SPM in Dx-5 MES-SA and their MDR positive human uterine sarcoma cells. To further examine the specificity of monofluorescent polyamine adducts for the polyamine transport system, the effect of pretreatment with poly-SPM (20 μM) on the uptake of FL-polyamines by PASMC was examined. The presence of poly-SPM significantly reduced the cellular accumulation of these FL-polyamine conjugates inside PASMC. These results indicate that the fluorescent polyamines are biologically equivalent to the native molecules, at least at the level of the transmembrane transport system.

In this study, we also examined the utility of CLSM analysis of fluorescein-labeled polyamines to localize polyamine binding sites within pulmonary artery smooth muscle cells. CLSM showed that after a 20-min treatment with either FL-SPM or FL-SPD, the fluorescence intensity was localized in the cytoplasm of the PASMC. There were clear cytoplasmic islands (of unconfirmed identity) which we suspect are mitochondria. We and others [1–4,9–17] have shown that the polyamine transport system in many mammalian cells is both energy- and temperature-dependent. Our studies demonstrated that the intracellular localization of FL-SPD or FL-SPM conjugates was abolished when cells were exposed to these conjugates at 4°C, a temperature at which the polyamine transport system is inoperative. These results further confirm the notion that the addition of a single fluorescent moiety does not significantly influence the rate of uptake of polyamines by PASMC. Further studies are in progress in our laboratory to examine the time-dependent distribution of FL-polyamines in living PASMC.

We also examined the distribution of FL-SPD and FL-SPM in both wild-type CHO cells and their polyamine transport deficient variants, CHOMGBG cells. As previously reported [26,27], parental CHO cells, but not CHOMGBG cells, express a temperature-dependent ^{14}C-SPD uptake system. As was seen in PASMC, the fluorescence intensity was localized in the cytoplasm of FL-polyamine-treated CHO cells. In addition, we found that the fluorescence intensity of both FL-SPD and FL-SPM was several-fold higher in the cytoplasm of CHO cells as compared to that detected in the cytoplasm of CHOMGBG cells, thus confirming the selectivity of FL-SPD and FL-SPM for the polyamine transporter.

This novel FL-polyamine/CLSM technique is useful for examining the cellular localization and distribution of polyamines. It may also be useful for the analysis of time-dependent changes in cellular polyamine distribution, particularly if they occur very rapidly. In addition, our technique might be employed for assaying changes in intracellular polyamine compartmentation depending upon the proliferative and phenotypic state of the cell.

Unlike polyamine metabolism and transport, where traditional biochemical, molecular and cellular approaches have generated substantial quantitative information, there is currently no method for tracking potential shifts in polyamines between intracellular compartments and quantifying such changes in terms of time and the functional state of the cell. Neither binding studies nor autoradiography are particularly well-suited for analysis of time-dependent changes in polyamine distribution, especially if they occur over a rapid time frame. Binding studies, though quantitative, are subject to artifacts caused by redistribution or leakage of the radiolabel that may occur during the cellular fractionation procedure. Autoradiography, while localizing the radiolabel, is not reliable quantitatively. Largely because of these shortcomings, the fundamental question of whether regulated polyamine compartmentation is a determinant of the functional state of the cell has remained unresolved. Therefore, one future application for our novel technique is to examine the time-dependent distribution of transported polyamines and to detect their ultimate cellular storage and binding sites.

Another future application of this methodology will be to provide an unprecedented opportunity to determine whether changes in intracellular polyamine compartmentation are regulated to affect the proliferative and phenotypic state of the cell. More specifically, to test whether the translo-

cation of fluorescent polyamines between specific intracellular compartments is engendered by stimuli promoting cellular proliferation or differentiation. The kinetics and mechanisms of alterations in intracellular polyamine compartmentalization will, for the first time, also be amenable to study.

In addition to the coupling of polyamines to a flourescein moiety, these natural substrates might also be linked to other fluorochromes, such as Texas red or dansyl chloride. Accordingly, in this study, a previously reported technique was used to prepare monodansylated SPD [9–13]. Polyamines are routinely derivatized with dansyl chloride to permit fluorescent detection after HPLC separation, which allows the subsequent quantification of cellular polyamine levels. Therefore, if labeling with dansyl chloride affords sufficient fluorescent intensity to permit microscopic localization, then it may be possible to perform complementary studies to both localize the polyamines and to assess their metabolism using the same population of cells. This will provide for an expeditious, two-step method for the complimentary determination of polyamine localization and metabolism. Our preliminary results (data not shown) suggest that dansylated polyamines are not as stable as FL-SPD, and decompose very rapidly. In addition, dansyl chloride failed to afford sufficient fluorescent intensity to be detected by CLSM or to permit microscopic localization of transported polyamines. Studies are in progress in our laboratory to develop a new analytical assay to quantify the cellular polyamine levels after their derivatization with fluorescein isothiocyanate. The combination of this assay method for polyamine detection with CLSM will provide a two-step method for the determination of the time-dependent distribution and metabolism of transported polyamines by biosynthetic enzymes.

5. Conclusion

In summary, the results of this study describe a novel method for visualizing the time-dependent intracellular distribution and organelle localization of polyamines in living cultured mammalian cells. Our results indicate that the fluorescein-labeled SPM and SPD molecules are taken up by cultured pulmonary artery smooth muscle cells in a manner analogous to unlabeled polyamines, and that confocal microscopy is useful for detecting the attached fluorescent label within specific structures of living PASMC. This precise analytical method will provide an unprecedented opportunity to determine whether changes in intracellular polyamine compartmentation are regulated to affect the proliferative and phenotypic state of the cell. Further, the kinetics and mechanisms of alteration in intracellular polyamine compartmentation will, for the first time, be amenable to study.

Acknowledgements

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